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ABSTRACT

BACKGROUND: The ability of bacterial cells to retain membrane integrity and membrane potential when challenged with Palladium (II) solution has not being examined previously, which would provide a platform towards the bio-fabrication of a multifunctional tandem bio-nanocatalyt. This study investigates the use of flow cytometry coupled with fluorescent probes to determine membrane integrity and membrane potential of cells of *Desulfovibrio desulfuricans* and *Bacillus benzeovorans* challenged with 1 mM of sodium tetrachloropalladate (II) (Na_2PdCl_4) solution at pH 2 followed by reduction of palladium (II) (Pd (II)) with formate to give 1wt% loading of Pd (0) on the cells.

RESULTS: Fluorescently labelled active bacterial cells retained over 80% of membrane potential when challenged with Pd (II) solutions except for *Bacillus benzeovorans* (Bb) with about 32% retention. Cell viability was also seen to be variable and strain-dependent while dead cells lack any membrane integrity. Since esterase activity is energy independent and unable to confirm the membrane potential of the bacterial cells, the dye 3, 3'-dihexyloxacarbocyanine iodide [DiO_6 (3)] was used to determine and confirm the membrane potential of the bacterial cells.

CONCLUSION: The results revealed that since fluorescently labelled bacterial cells containing Pd (0) can retain metabolic activity when analysed with flow cytometry, it provides the potential for combining chemical catalysis with biochemical activity in reactions that require metabolic synergy.

Key words: Catalysis; Cell viability; Flow cytometry; Membrane integrity; Membrane potential; Palladium.

INTRODUCTION

Flow cytometry has widespread applications in biotechnology, industrial and medical research.[1-4] It provides qualitative and quantitative analytical measurement of individual cells using fluorescence emission of labelled cells when they pass individually through a light source (*e.g.* laser).[5-7]

Cell viability determination plays a strategic role particularly in, for example food microbiology and microbial pharmacology.[1] The flow cytometry methods depend on the uptake by dead or non-viable cells of dyes that are normally excluded by viable cells or those with intact membranes. Propidium iodide (PI) for instance is a membrane impermeable molecule (~ 668 Da) with the ability to bind to nucleic acid only following the loss of cellular integrity in dying, dead or necrotic cells, producing red fluorescence, as PI cannot cross an intact cytoplasmic membrane.[8] Fluorescein diacetate (FDA) is a non-fluorescent dye but when taken up by esterase-

active cells it is hydrolysed by a non-specific intracellular esterase to produce green fluorescence that is retained by cells with intact cell membranes.[9] A charged and slightly lipophilic dye like 3, 3'-dihexyloxacarbocyanine ($DiOC_6$ (3)) has been used to determine the membrane potential of *E. coli* as a measure of cell viability, resulting in green fluorescence in intact cells with polarised membranes,[10] while anionic dyes will interact with depolarized membranes.[11] There are other dyes known for their relevance in the determination of cell viability [12, 13] and these can be used in combination with others or conjugated with antibodies in most cases. Appropriate dyes can be used to quantify the effects of stress or toxic chemicals on the cytoplasmic membrane integrity and physiology of a bacterial population.[14-16]

Using flow cytometry, cell viability measurement has been applied in the rapid enumeration of probiotic bacteria, [17] pathogenic bacteria, [18] root-associated bacteria, [19] and *Lactobacillus rhamnosus* in chocolate.[20] Other parameters such as light scattering, DNA, RNA, protein contents, identification of antibody-antigen interaction, altered cell states such as apoptosis and cell death [1, 7] can also be measured.

Studies on cells 'decorated' with metallic nanoparticles (NPs) such as Pd (0) have been widely reported using various bacterial strains.[21- 23] However catalytically active materials synthesized using these bacteria have mostly been produced in dry powdered form following drying of the metallized cells. A recent study has shown that, contrary to reports that Pd-NP (palladium nanoparticle) localization is restricted to the surface layers, [24, 25, 22] cells of *Bacillus benzeovorans*, *Desulfovibrio desulfuricans* [26] and *Escherichia coli* [27] have shown extensive

deposition of small intracellular Pd-NPs which implies a mechanism of cellular 'trafficking' of the Pd (II) initially applied. 'Next generation' biocatalysis would involve 'tandems' whereby both Pd-NPs and biochemical processes contribute to single cell 'nanofactories' as illustrated by Foulkes et al.[28] in a deracemization reaction whereby application of palladized cell suspension of engineered *E. coli* was used for the sequential oxidation and reduction coupled with biotransformation of 1-methyltetrahydroisoquinoline (MTQ). De Windt *et al.,* [29] reported the determination of cell viability of Pd-challenged cells of *Shewanella oneidensis* using flow cytometry. However, to our knowledge, there are no in-depth studies on the determination of both cell viability and membrane integrity of palladium-challenged cells and this forms the major focus of this study. This study reports the viability and membrane integrity of "palladized" cells of Gram-positive *Bacillus benzeovorans* NCIMB 12555 and two strains of Gram-negative *Desulfovibrio desulfuricans* (NCIMB 8307 and NCIMB 8326) which have shown different patterns of Pd-deposition.[26] Therefore, the main objective of the study was to compare the viabilities of Pd-challenged cell suspensions through the measurement of membrane integrity and membrane potential as indices of cell viability using flow cytometry. This would underpin a new direction in the synthesis of potentially novel catalysts which combine biochemical and chemical catalysis in single cells.

MATERIALS AND METHODS

Bacterial growth and cell preparation

Gram-negative *Desulfovibrio desulfuricans* ('Dd') (NCIMB 8307 and NCIMB 8326) were grown anaerobically based on the method of Deplanche et al.[25] in Postgate's C medium while Gram-positive *Bacillus benzeovorans* ('Bb') (NCIMB 12555) was grown aerobically in a nutrient medium.[26] Cells were harvested by centrifugation during exponential growth, concentrated in a small amount of MOPS (4-morpholinepropanesulfonic acid)-NaOH buffer, pH 7 and stored under oxygen-free nitrogen (OFN) at 4^{o}C until use, usually within 24 h.

Pd (II) –challenge of cells and reduction of Pd (II) with formate

Harvested cell suspensions (20 mg/ml) were estimated based on predetermined OD_{600} and dry weight conversion for *D. desulfuricans* [30] and *B. benzeovorans* [31] and challenged with 1 mM Pd (II) solution of Na_2PdCl_4 (pH 2) to make a final 1wt% Pd loading on cells. Samples were divided into two; a Pd-cell suspension was supplemented with 1 M sodium formate (final concentration of 20 mM) for 1 h with shaking and the control was without formate. Control comprised cells suspended at pH 2 but not supplemented with Pd (II) and cells killed by heat treatment (70^{o}C; 1 h). Membrane integrity and membrane potentials were then determined by flow cytometry.

Measurement of membrane integrity and membrane potential *via* flow cytometry

Cell samples (5 ml) each were divided into four: Pd (II)-challenged cells (without formate), Pd-challenged cells with Pd (II) reduced using formate, Pd-free live cells and heat-killed cells (without Pd and formate) as controls. After incubation as above and prior to flow cytometry

measurement, the cells were washed by centrifugation (12, 000 x g, 15 min, 4^{o}C) to remove unbound metal ion and MOPS-NaOH buffer and then re-suspended in ice cold (10 ml) phosphate buffered saline (PBS) (10 mM PBS, containing NaCl (8g), KCl (0.2g), Na_2HPO_4 (1.44g), KH_2PO_4 (0.2g) per L), pH 7.4, washed twice (12, 000 x g, 15 min, 4^{o}C) and 0.1 ml of each type of cell was resuspended to approx 10^6 cells/ml in PBS (1.9 ml) to a final volume of 2 ml.[15] For determination of membrane integrity and esterase activity, solutions of fluorochromes; fluorescein diacetate (FDA) (40µl, 0.1 mg/ml aq) and of propidium iodide (PI) (4µl, 1 mg/ml aq) were added simultaneously to the 2 ml cell mixture. Meanwhile, for the measurement of membrane potential, 3, 3'-Dihexyloxacarbocyanine iodide [$DiOC_6$ (3)] (20µl, 1 µM) was added to a separate sample. Mixtures were stored in the dark for 15 minutes at room temperature. Flow cytometry measurements were taken immediately in triplicates using a FACSCanto II flow cytometer, Becton Dickson (San Jose Palo Alto, California) equipped with three lasers: 488 nm blue, 620 nm red and 405 nm UV. Samples were measured in a green fluorescence detector or filter, FL1 (fluorescein isothiocyanate, FITC) and a red fluorescence detector or filter, FL2 (phycoerythrin-propidium iodide, PE-PI) mode in logarithmic scale channels at medium speed. The band pass filters used were 530 nm and 580 nm and results were analysed using BD Diva 6.1 software. Over 10,000 events were registered per analysis.

Statistical analyses

Statistical analysis was performed using Origin 7.5 software. The statistical differences and significance were assessed using ANOVA test and $P < 0.05$ was considered significant.

RESULTS

Evaluation of membrane integrity of Pd-challenged cells

Initial measurements were made to differentiate between viable, non-viable and damaged cells (i.e. neither viable nor yet dead) based on double staining with the dyes flourescein diacetate (FDA) and propidium iodide (PI). Four test samples of Dd 8307, Dd 8326 and Bb (Fig 1, Table 1) were examined: Pd-free live cell controls, Pd (II)-sorbed cells (no-formate), reduced Pd (II) (with formate) and heat-killed cell controls. In the case of Pd (II)-treatment, a 1mM Na_2PdCl_4 of 1% Pd (II) loading on cells was used with 20 mM final formate concentration as an electron donor to reduce Pd (II) in cells that had been allowed to take up Pd (II) for 45 min before formate addition. The work was done at pH 2 because an acidic optimum was previously established (Yong et al., 2002)[32] for the catalytic synthesis of biogenic Pd (0) with optimal catalytic activity.

The results of cell viability analysis are interpreted based on the percentage of each sub-population in bivariate dot plots (Fig. 1). Viable cells, defined here as having intact membranes and esterase activity (PI^- FDA^+, lower-right quadrant) are indicated in Fig. 1 in green. Dead or non-viable cells, defined as having non-intact membranes (PI^+ FDA^-, upper-left quadrant) are shown in red. Damaged cells, defined as having some permeability to PI but retaining some green fluorescence due to FDA (PI^+ FDA^+, upper-right quadrant) are shown in dark-blue.

Fluorescence due to noise or background (lower-left quadrant) is shown in light blue. With the Pd-free live cell controls, viable cells with intact cell membranes and esterase activity are shown in green (Figure 1a, b, c). The population of viable cells in Pd-free live cells was 95- 100% (Table 1) with no dead cells detected. The proportion of damaged cells in Dd 8307 and Bb was significantly lower ($P < 0.05$) than Dd 8326 when cells were challenged with Pd (II) (Table 1). When Pd (II) was reduced on the cells using formate as electron donor, the cell viability was not significantly ($P > 0.05$) reduced when compared to Pd-free live cells except for Dd 8326 (Fig 1i) with a population of 35.3% viable cells and 52.5% damaged cells ($P < 0.05$) (Table 1) at 0.05 significance level. In contrast, no non-viable cells were detected in Bb and Dd 8307 although 12.1 % were found in Dd 8326. In the heat-killed cell controls > 99% of the cell population of Bb, Dd8307 and Dd8326 were dead or non-viable (Table 2) indicated in red (Figs 1j, k, l) with <1% sub-population of damaged cells.

Assessment of membrane potential of Pd-challenged cells

Membrane potential is an alternative way to compare the responses of Pd-challenged cells using cationic 3, 3'-dihexyloxacarbocyanine iodide [$DiOC_6$ (3)] dye which interacts with intact cells (polarised membranes) but not permeabilized, dead or resting cells which have depolarised membranes.[11] Apart from metal-free live cell controls, cell samples were first treated with Pd (II) then with dye. There was no heat-killed control as dead cells do not have membrane potential (the cationic dye does not interact with depolarised cell membranes.[11] The results of cell viability (membrane potential) are described in terms of active and inactive state of the cell

membrane due to interaction of the dye with lipophilic sites within the membrane (Table 2) while the fluorescence histograms (Fig 2) show the metabolic state of the cell membrane as active (P2) or inactive (P3). P2 and P3 are event markers, designating positive and negative events respectively (Fig 2).

The Pd-free live cells (positive control) of Bb, Dd8307 and Dd8326 showed that about 100%, 99.7% and 98.9% of the cell population were metabolically active with less than 2% of cells being inactive (Fig 2a, b, c and Table 2). When challenged with Pd (II), the membrane activity of Bb reduced to 32.1% and over 60% of the cell population were inactive (Fig 2d, Table 2). With Dd8307 and Dd8326, the number of cells with intact membranes reduced to 88.8% and 94.0% respectively (Figs 2e, f and Table 2) with a correspondingly increased number of inactive cells. However, when Pd (II) on the challenged cells was reduced with formate to Pd (0) in all three strains, the membrane potential of the cells was sustained and there was no significant difference when compared with Pd-free live cells (Figs 2g, h, i) while the number of inactive cells was also low (Table 2).

DISCUSSION

This study compared the viability of Pd-challenged cells of two strains of Gram-negative *Desulfovibrio desulfuricans* (Dd8307 and Dd8326) and a Gram-positive *Bacillus benzeovorans* (Bb) by measuring membrane integrity and membrane potential. The double staining of cells

with fluorescein diacetate (FDA) and propidium iodide (PI) was used to determine membrane integrity and differentiated between viable and non-viable cells.[8] A slight decrease in the population of viable cells when challenged with palladium (II) (Table 1) due to its toxicity was more pronounced in *D. desulfuricans* (Dd8326) where only 9.51% of the cell population remain viable. Notably, over 80% of the cells were 'damaged', i.e. these cells were neither viable nor dead.[9] In contrast, the other two strains appeared more resistant to Pd toxicity. An alternative explanation for this significant ($P < 0.05$) effect on Dd8326 could be that, while *Desulfovibrio* spp. are strict anaerobes some of them are more tolerant to O_2 than others [33, 34] and during flow cytometry analysis, the cells were unavoidably exposed and analysed in air which could have resulted in additional stress or injury.[35] However, there was an overall improvement in the viability of the cells (Figs 1g, h, i) when the Pd (II) was reduced in the presence of formate as an electron donor (especially in Dd 8326; Fig 1i, Table 1). This suggests that the stress suffered by this organism was Pd (II)-related and that the formation of Pd (0) ameliorates this toxicity, although whether this is a metabolic detoxification via reductive conversion of Pd (II) to Pd (0), thereby reducing ongoing Pd (II)-related oxidative damage, is not known. Moreover, other work using acid-tolerant bacteria (background described in Eguchi and Utsumi and Pennacchietti *et al.,*) [36, 37] showed that the Pd (0) deposition and catalytic activity of the resulting 'bio-Pd (0)' was indistinguishable in parental and acid-resistant cells of *E. coli* (L. Zajac, A.J. Murray, P.A. Lund and L.E. Macaskie, unpublished). In addition, studies have shown that ionic gold is more toxic than gold nanoparticles.[38]

Redox-active metals like Ni promote oxidative damage as they can exist in the (II) and (I) states; [39] Pd (II) can possibly act as a 'surrogate' for Ni (II) (they are in the same group of the periodic table) but not substitute functionally. Some similarities in biochemical 'processing' of Ni (II) and Pd (II) might be expected, e.g. they are known to cross-react as sensitisers, although very little comparative information is available.[40] It is well known that, in general, metallic ions have the ability to bind to DNA, enzymes or proteins, exhibiting cytotoxic or genotoxic effects.[41] This was exemplified by Boswell *et al.,*[14] who used flow cytometry to study the effects of some heavy metals on *Acinetobacter johnsonii*, which revealed permeabilized cytoplasmic membranes, as confirmed by transmission electron microscopy. The actual mechanism of metal toxicity is beyond the scope of this work, while comparison with other work is complicated since here the cells were placed under the additional pH stress. However, in this study, once reduced to Pd (0) the dualstress had no effect on the viability of Bb and Dd8307 and the small effect observed in Dd8326 may be associated with a greater toxicity of Pd (II). Note also, however, that since the Pd (II) challenge was done at pH 2 metal toxicity may have been differentially augmented by acid-stress although, again this does not account for the recovery of the cells once Pd (II) was reduced to Pd (0). According to De Windt *et al.,*[29] as we report here, Pd (II) treated cells (without electron donors) of *Shewanella oneidensis* showed a 60% reduction of cell viability while the addition of hydrogen or formate to Pd (II) challenged cells reduced the toxicity of Pd (II) on the cells without significantly reducing cell viability. However, the addition of formate proved to be less effective in reducing Pd (II) toxicity than hydrogen. Since the pK_a of

formic acid is 3.75 and undissociated formic acid can enter cells, dissociating within the cell to H^+ and COO^-,[42, 43] intracellular acidification leads to cell death.[44] Fermentative bacteria (and some *Bacillus* spp) have effective formate hydrogen lyase activity to effect formate breakdown; this enzyme has been well-reported in *D. desulfuricans* but its differential expression in the two *Desulfovibrio* strains would not account for the recovery seen following reduction of Pd (II) to Pd (0) which suggests that the effects observed are attributable to the free Pd (II) species, either as free Pd^{2+} or the $PdCl_4^{2-}$ that predominates in a chloride solution.[45] The cell membrane contains organic compounds, mainly phospholipids, and also proteins and attached polysaccharide chains [46] and oxidative attack on various functional groups of the membrane may be a key mechanism leading to heat-induced cell death [47] and lipids are the most vulnerable. In support of this, studies using XPS (a surface technique, which probes the outer membrane ~5-10 nm) have shown an effect of Pd on cells of *E. coli* [27] while some reduction of Pd (II) in the absence of added electron donor was observed using *D. desulfuricans* (NCIMB 8307 and NCIMB 8326) suggesting a function of Pd (II) as an oxidant (J.B. Omajali and L.E. Macaskie, unpublished). Examination by XPS revealed no Pd(I) but this does not preclude its formation in the cellular regions below the outermost surface accessible by this method .

The metabolic or membrane potential of the cell is very important to cell viability. There was no significant difference in the membrane potential of the Pd-free live cells of the three bacteria when compared to cells containing reduced Pd (0) (Table 2). Intracellular palladium nanoparticles were synthesized *via* formate and hydrogen reduction in all three strains but it

could not be proved that membranes were intact.[26] However, the present study shows that, even though ~90% of the Pd (II)-sorbed cells of Bb were viable, the metabolic activity of these cells was low with ~68% of the population in an inactive state. In contrast, even though the population of viable cells of Dd8326 (Table 2) was low, over 90% of the cell population were still metabolically active. This suggests that the distinction between life and non-life is not absolute as many cells may exist in a dormant state [48] but may give a viable direct count.[49] Hence, most of the cells that appeared non-viable in Dd8326 could still carry out metabolic functions while the sub-population of Pd (II)-challenged cells of Bb that appeared viable was not active metabolically. A similar response, overall, was given by both strains of *D. desulfuricans*; these Gram-negative cells contained both outer membrane and periplasmic capabilities which are absent in Gram-positive cells and speculations as to the root of the observed differences is unwarranted.

CONCLUSION

The ability of *Bacillus benzeovorans* and two strains of *Desulfovibrio desulfuricans* to exhibit intact cell membrane and maintain metabolic activity when challenged with 1mM Pd (II) solution may be relevant for the development of tandem catalysts that combine biotransformation and chemical catalysis reactions. While adverse effects were seen in *B. benzeovorans* and *D. desulfuricans* 8326 on challenge of the cells with Pd(II) its reduction to Pd(0) restored cell viability, membrane integrity and membrane potential. Currently the choice of bacterial 'factory' to make Pd-nanoparticle catalyst is based on trial and error. Within a single species (*D. desulfuricans*) a greater effect of Pd(II) on cell viability accompanied subsequent production of a less effective bio-metallic catalyst. Further studies are warranted to establish whether flow cytometry has potential as a rapid screen for the most promising strains, without the need to identify which precise biochemical mechanisms are responsible for trafficking and deposition of catalytically-active Pd(0).

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Legends to Figures

Figure 1. Flow cytometry analysis of cell viability (membrane integrity and esterase activity). *B. benzeovorans* (Bb), *D. desulfuricans* (Dd8307 and Dd8326) cells were stained with propidium iodide and FDA; data are shown in bivariate dot plots with FDA fluorescence on the X axis and PI fluorescence on the Y axis. Populations are coloured according to metabolic state; viable cells in green, non-viable cells in red, damaged cells in dark blue and noise or background signals in light blue. Pd-free live cells (a, b, c); Pd (II)-sorbed cells (d, e, f); Reduced Pd (II)[bio-Pd(0)] on cells (g, h, i) and heat-killed cells (j, k, l).

Figure 2. Fluorescence histograms showing membrane potential of Pd-challenged cells of *B. benzeovorans* (Bb), *D. desulfuricans* (Dd8307 and Dd8326) with two gates: P2 designating cells with active membrane potential; and P3 for cells with inactive membrane potential. Cell samples were divided into Pd-free live cells (a, b, c); Pd (II)-sorbed cells (d, e, f) and reduced Pd (II) (with formate)

on cells (g, h, i). X-axis is $DiOC_6$ (3) fluorescence. Y-axis is increase in cell count or population. P2 and P3 were determined directly from the flow cytometer.

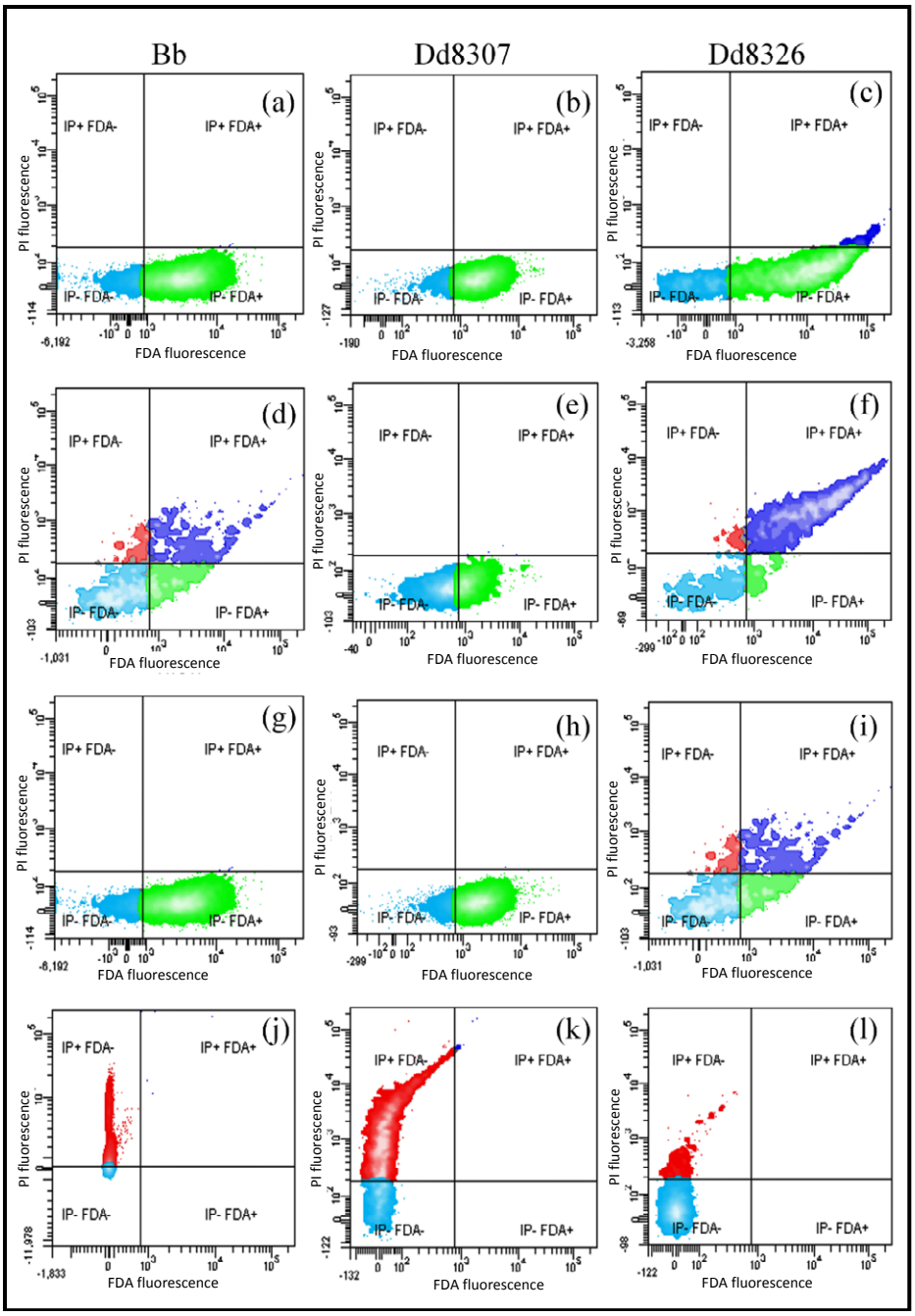

Figure 1

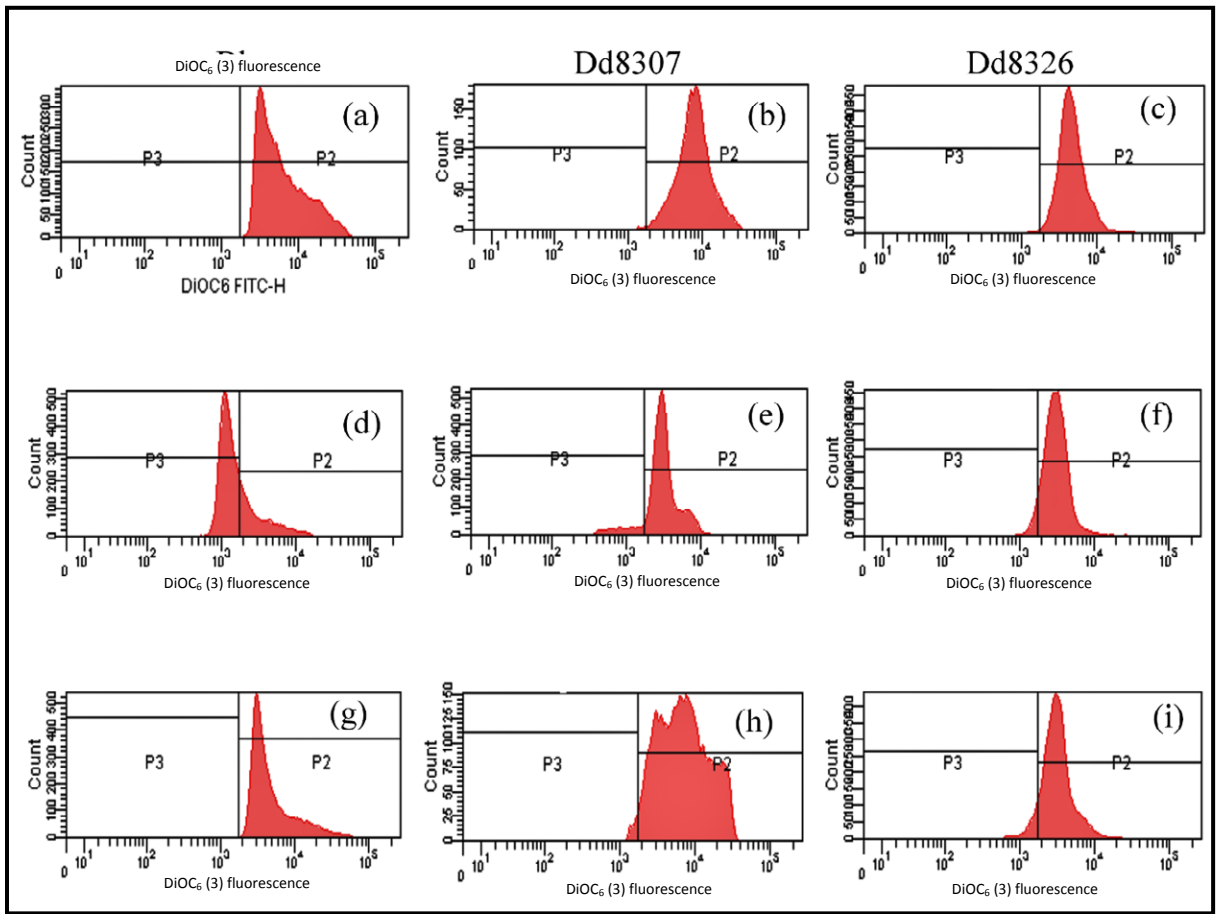

Figure 2

Table 1 Determination of membrane integrity and esterase activity using flow cytometry

		Cell viability (%)		
Test samples	State of cells	Bb	Dd8307	Dd8326
Pd-free live cells	viable	99.90 ± 0.10	100 ± 0.00	95.33 ± 0.50
	non-viable	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	damaged	0.16 ± 0.16	0.00 ± 0.00	4.71 ± 0.51
Pd (II)-sorbed cells	viable	89.29± 2.06	99.30 ± 0.33*	9.51 ± 1.32
	non-viable	0.55 ± 0.11	0.00 ± 0.00	3.85 ± 0.22
	damaged	10.12 ± 1.92	0.36 ± 0.19	86.64 ± 1.17
Reduced (bio-Pd (0) on cells)	viable	99.89 ± 0.04*	99.93 ± 0.07*	35.30 ± 1.15
	non-viable	0.00 ± 0.00	0.00 ± 0.00	12.13 ± 1.31
	damaged	0.15 ± 0.04	0.00 ± 0.00	52.55 ± 1.52
Heat-killed cells	viable	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	non-viable	99.54 ± 0.19	99.83 ± 0.00	99.91 ± 0.09
	damaged	0.42 ± 0.20	0.17 ± 0.17	0.04 ± 0.04

Results are replicates of three separate determinations, Mean ± SE.

Cells were treated with 1wt% solution of Na_2PdCl_4 (1mM Pd (II)). Reduction of Pd (II) solution onto bacterial cells was done with 20 mM sodium formate as electron donor. Bb: *Bacillus benzeovorans*, Dd8307: *Desulfovibrio desulfuricans* NCIMB8307 and Dd8326: *Desulfovibrio desulfuricans* NCIMB 8326. Samples were stained with flourescein diacetate (FDA) and propidium iodide (PI) resulting in green and red fluorescence for viable and non-viable cells respectively. *$P < 0.05$.

Table 2 Determination of membrane potential of cells using flow cytometry

		Membrane potential of cells (%)		
Test samples	Metabolic state	Bb	Dd8307	Dd8326
Pd-free live cells	active cells	100 ± 0.00	99.73 ± 0.00	98.87 ± 0.41
	inactive cells	0.27 ± 0.03	0.27 ± 0.03	1.13 ± 0.41
Pd (II)-sorbed cells	active cells	32.05± 1.15	88.83 ± 2.37	94.03 ± 0.42*
	inactive cells	67.95± 1.15	11.17 ± 2.37	5.97 ± 0.42
Reduced (bio-Pd (0) on cells)	active cells	99.97 ± 0.03*	98.70 ± 0.40*	94.87 ± 1.47*
	inactive cells	0.03 ± 0.03	1.30 ± 0.40	5.90 ± 0.90

Results are replicates of three separate determinations, Mean ± SE.

Cells were treated with 1wt% solution of Na_2PdCl_4 (1mM Pd (II)). Reduction of Pd (II) solution onto bacterial cells was done with 20 mM sodium formate as electron donor. Bb: *Bacillus benzeovorans*, Dd8307: *Desulfovibrio desulfuricans* NCIMB8307 and Dd8326: *Desulfovibrio desulfuricans* NCIMB 8326. Interaction of an intact cell membrane with the dye, 3, 3'-dihexyloxacarbocyanine iodide [DiO_6 (3)] verifies membrane potential of the cells. *$P < 0.05$.